

RESOURCE ARTICLE

Trio-binned genomes of the woodrats *Neotoma bryanti* and *Neotoma lepida* reveal novel gene islands and rapid copy number evolution of xenobiotic metabolizing genes

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Abstract

The genomic architecture underlying the origins and maintenance of biodiversity is an increasingly accessible feature of species, due in large part to third-generation sequencing and novel analytical toolsets. Applying these techniques to woodrats (*Neotoma* spp.) provides a unique opportunity to study how herbivores respond to environmental change. *Neotoma bryanti* and *N. lepida* independently achieved a major dietary feat in the aftermath of a natural climate change event: switching to the novel, toxic food source creosote bush (*Larrea tridentata*). To better understand the genetic mechanisms underlying this ability, we employed a trio binning sequencing approach with a *N. bryanti* × *N. lepida* F₁ hybrid, allowing the simultaneous assembly of genomes representing each parental species. The resulting phased, chromosome-level, highly complete haploid references enabled us to explore the genomic architecture of several gene families—cytochromes P450, UDP-glucuronosyltransferases (UGTs), and ATP-binding cassette (ABC) transporters—known to play key roles in the metabolism of naturally occurring toxic dietary compounds. In addition to duplication events in the ABCG and UGT2B subfamilies, we found expansions in three P450 gene families (2A, 2B, 3A), including the evolution of multiple novel gene islands within the 2B and 3A subfamilies, which may have provided the crucial substrate for dietary adaptation. Our assemblies demonstrate that trio binning from an F₁ hybrid rodent effectively recovers parental genomes from species that diverged more than a million years ago.

KEYWORDS

adaptive evolution, dietary adaptation, functional genomics, genome sequencing, metabolic adaptation, xenobiotic metabolism

1 | INTRODUCTION

At every meal, mammalian herbivores confront the possibility of being poisoned by their food (Dearing et al., 2005). In their seminal work on dietary strategies, Freeland and Janzen (1974) predicted that liver biotransformation enzymes dictated detoxification ability and, therefore, diet breadth in herbivores. Despite decades of

pharmacological research on laboratory species and humans, we know relatively little about the mechanisms used by mammalian herbivores to metabolize plant toxins or adapt to new diets (Dearing et al., 2005; Foley et al., 1999; Marsh et al., 2006; Moore et al., 2015; Sorensen et al., 2006; Sorensen & Dearing, 2006). Nevertheless, this fundamental information on how mammalian herbivores metabolize dietary toxins is critical in understanding the evolution of dietary

shifts that entail exploiting new food sources, or shifts that are imposed by flora changing in response to natural and anthropogenic climate change. By studying woodrats (genus *Neotoma*), which include many species that have weathered environmental change (Smith et al., 1995) and adapted their diets to include novel toxic plants (Nielsen & Matocq, 2021), we can gain insight into both of these areas.

Woodrats are the sister genus to *Peromyscus* (Figure 1a), and are endemic to North and Central America (Hall, 1982). Though most *Neotoma* species have distinct ranges, in some areas, closely related species come into contact and produce fertile hybrids (Coyner et al., 2015; Patton et al., 2007; Shurtliff et al., 2014). Around 17,000 years ago, the region that is now the southwestern United States experienced a temperature increase as the Late Glacial Cold Stage ended. This caused substantial disruption to the vegetation throughout the region and resulted in the establishment of the arid deserts of the American Southwest (Van Devender, 1977; Van Devender & Spaulding, 1979; Wells & Woodcock, 1985). In the wake of this climate change event, juniper (*Juniperus* spp.) (Figure 1b) populations declined, and large portions of their former range were replaced with what ultimately became the dominant shrub of the region: creosote bush (*Larrea tridentata*) (Figure 1c) (Van Devender, 1977; Van Devender & Spaulding, 1979; Wells & Hunziker, 1976). This vegetation shift forced resident woodrat species to switch from a diet of juniper and cactus to one containing significant quantities of creosote. These food sources have radically different plant secondary

compounds and presumably exerted disparate selective forces on the hepatic detoxification systems of *Neotoma bryanti* (Figure 1d) and *N. lepida* (Figure 1e) (Dearing et al., 2005; Holchek et al., 1990; Mabry et al., 1977). If selection for this novel diet was facilitated by adaptation, then evidence for this is likely to be found in the overall structure of the xenobiotic metabolizing genes in both species.

Identifying and characterizing the putative genetic basis of dietary adaptations demands high-quality and annotated reference genomes; however, these resources were lacking for *Neotoma* woodrats. Prior to this study, a draft genome for *N. lepida* (Campbell et al., 2016) was the sole genomic reference available for the genus, and, while nearly complete, it is highly fragmented (scaffold N50 of 151 kb), thereby limiting genetic analyses. To address this deficiency, we generated new genomic resources for both *N. lepida* and *N. bryanti*. These two woodrat species are sister taxa with an estimated 1.5-million-year divergence time (Patton et al., 2007). The desert woodrat (*N. lepida*) is found across many habitats in the western United States, from as far north as Idaho and Oregon down through Utah and eastern California. In contrast, Bryant's woodrat (*N. bryanti*) has a smaller range primarily in the coastal woodlands and chaparrals of southern California, with some populations extending into desert habitats (Patton et al., 2007). Both species have populations that feed on creosote bush (Weinstein et al., 2021). The ranges of these two species are largely distinct, but in the few locations where they overlap (e.g., Morongo Valley, California), hybrids occur (Patton et al., 2007). We leveraged the ability of these two

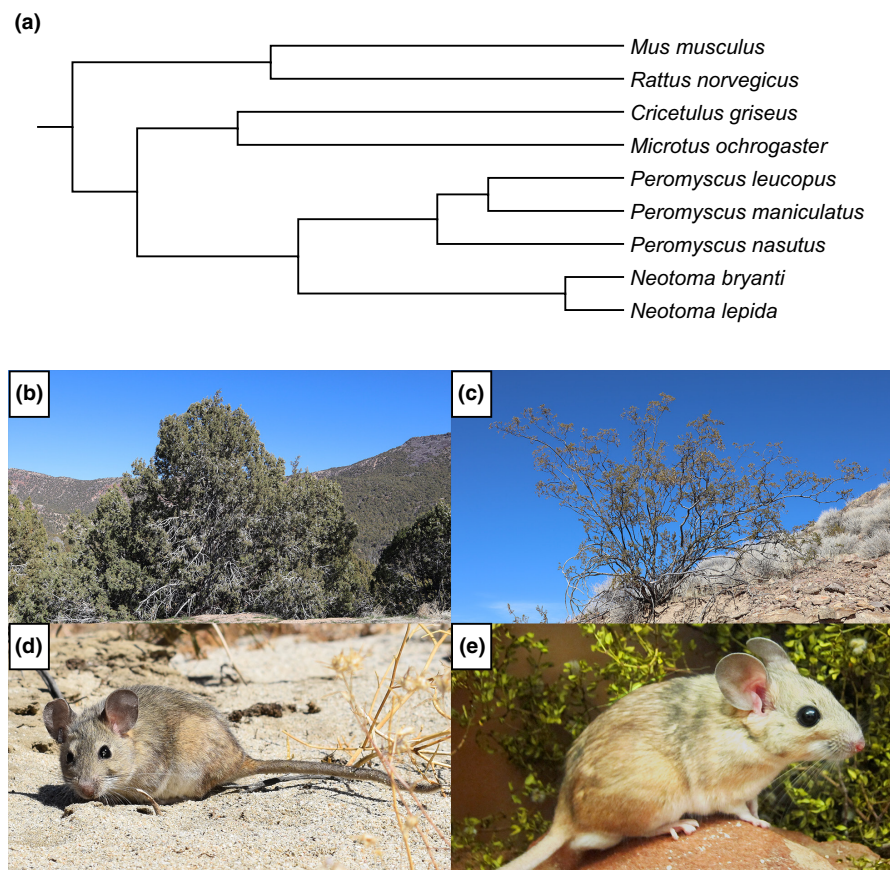


FIGURE 1 Phylogeny of Cricetidae rodents, common dietary items, and photographs of *Neotoma bryanti* and *Neotoma lepida*. (a) *Neotoma* woodrats are closely related to deer mice of the genus *Peromyscus* and more distantly related to other members of the family Muridae. The phylogeny for this panel was downloaded from <http://www.timetree.org> and visualized in MEGA X (Stecher et al., 2020). (b,c) Images of juniper (*Juniperus* spp.) (b), an ancestral *Neotoma* food source, and creosote bush (*Larrea tridentata*) (c), the shrub that replaced it in many low elevation localities in the Mojave Desert. (d,e) Photographs of *N. bryanti* (d) and *N. lepida* (e), the two woodrat species selected for the trio binning approach

species to hybridize and applied the recently developed trio binning approach implemented in *CANU* (Koren et al., 2018) to an F_1 cross between *N. bryanti* and *N. lepida* in order to simultaneously generate haploid reference genomes for both species. This approach combines PacBio long read sequencing of the hybrid individual with Illumina short read sequencing of the parental individuals. *k*-mers identified in the Illumina read data are used to bin the hybrid reads by parent-of-origin, and each parental haplotype is then assembled separately from the binned PacBio reads. By using this method, we avoided some of the issues that plague the reconstruction of diploid vertebrate genomes, while also keeping sequencing and computational costs low. Although this technique has previously been used to generate genome assemblies for hybrid species within the Bovidae (Rice et al., 2020) and Felidae (Bredemeyer et al., 2020) families, this marks the first time this approach has been used to generate genomes for two rodent species and for two species of naturally hybridizing mammals.

The resulting reference genomes are assembled to the chromosome level, and their sequence content and annotations are highly complete by multiple metrics. To better understand the genomic basis of adaptation to different toxic diets, we interrogated the genetic architecture and possible expansion dynamics of several xenobiotic metabolizing gene families, with a particular focus on the cytochromes P450 (henceforth P450) 2A, 2B, and 3A genes. The P450 enzymes produced by these genes are critical in the biotransformation of toxins like those present in creosote bush and juniper (Huo et al., 2017; Magnanou et al., 2009; Shah et al., 2016; Skopec et al., 2013; Wilderman et al., 2014). Novel dietary niches involving toxic food are hypothesized to exert strong selection pressure for herbivore defence against potential toxic effects (Dearing et al., 2005), and previous work in these woodrats and other herbivorous mammals suggests that the evolution of gene copies, specifically those in P450 subfamily 2B, play an important role in dietary adaptation (Kitanovic et al., 2018; Magnanou et al., 2009; Malenke et al., 2012). Therefore, we expected to document copy number expansion of the 2B and potentially other P450 gene subfamilies in comparison to other rodents.

2 | MATERIALS AND METHODS

2.1 | Animal collection and handling

The female *N. bryanti* used in the F_1 cross was captured in March 2018 near Pioneertown, California, USA (lat. 34.151, long. -116.479). The male *N. lepida* was captured in October 2018 near Kelso Depot, Mojave Preserve in California, USA (lat. 35.009, long. -115.645). *Neotoma lepida* individuals ($N = 4$) used for the transcriptomics portion of the work were captured in March 2018 in Lytle Ranch Preserve, Utah, USA (lat. 37.070, long. -114.000). Woodrats were live-trapped at their respective sites using $7.6 \times 8.9 \times 22.9$ cm Sherman traps (H. B. Sherman Traps, Inc., Tallahassee, FL, USA). The *N. lepida* captured near Kelso Depot was obtained under the

California Department of Fish and Wildlife permit number SC-2105, MOJA-00038 issued to James Patton, while all other animals were trapped under the California Fish and Game permit SCP-8123 and Utah Game and Fish permit 1COLL5194-2 issued to M. Denise Dearing. Animal work was approved under the University of Utah's Institutional Animal Care and Use Committee (Protocol 16-02011).

After transport to the University of Utah's School of Biological Sciences animal facility, animals were housed individually (except for mating) in $48 \times 27 \times 20$ cm shoebox cages. The facility was maintained at 24°C, 15%–20% relative humidity and with a 12-h light/dark cycle. Animals were provided rabbit chow (Teklad formula 2031; Envigo, Indianapolis, IN, USA) and water ad libitum. The *N. bryanti* \times *N. lepida* cross yielded three hybrid individuals (two females, one male) in March 2019, and one female from this litter was selected for sequencing to ensure the X chromosome could be reconstructed for each haplotype.

Animals were dispatched prior to dissection. For DNA sequencing, liver tissue was extracted and stored at -80°C. For RNA sequencing, liver tissue was placed in RNeasy Lysis Buffer (Qiagen) prior to storage at -80°C.

2.2 | Genomic library preparation and sequencing

For the F_1 parents, DNA was extracted from liver tissue using the DNeasy Blood & Tissue Kit (Qiagen); the concentration (~70 ng/ μ l) was validated using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Following shearing with a Covaris S2 Focused-ultrasonicator (Covaris), libraries with an average insert size of 450 bp were prepared by the University of Utah's Huntsman Cancer Institute High-Throughput Genomics Core Facility using the Illumina TruSeq DNA PCR-Free Library Prep Kit (Illumina). 2×150 bp reads for each parent were generated on an Illumina NovaSeq 6000 instrument using the NovaSeq S2 Reagent Kit (Illumina). 67.44 \times and 81.10 \times coverage for the *N. bryanti* mother and *N. lepida* father, respectively, were obtained based on a genome size estimate of 2.4 Gb derived from the previous *N. lepida* ASM167557v1 assembly (Campbell et al., 2016).

For the hybrid offspring, DNA extraction, library preparation and sequencing were carried out by the DNA Sequencing Center at Brigham Young University. Following DNA extraction from liver tissue with the Qiagen MagAttract HMW DNA Kit (Qiagen), libraries were generated using the SMRT Bell Template Preparation Kit (Pacific Biosciences), and sequencing was performed using eight SMRT Cells on a PacBio Sequel II instrument (Pacific Biosciences). This yielded 787.76 Gb of sequence data, corresponding to approximately 328 \times coverage.

2.3 | RNA library preparation and sequencing

RNA was extracted from liver tissue using the RNeasy Mini Kit (Qiagen). The concentration (~75 ng/ μ l) and RNA quality

(RIN ≥ 9.0) for each sample was validated with a Qubit RNA BR Assay Kit (Thermo Fisher Scientific) and Agilent Technologies RNA ScreenTape Assay (Agilent Technologies), respectively, before library preparation and sequencing was carried out by the University of Utah's Huntsman Cancer Institute High-Throughput Genomics Core Facility. Libraries were generated using the Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina), and 2×150 bp reads were produced on an Illumina NovaSeq 6000 instrument using the NovaSeq S2 Reagent Kit (Illumina). For sample counts, see [Table S1](#).

2.4 | Mitochondrial genome assembly

Illumina reads for each parent were imported into CLC GENOMICS WORKBENCH v.12 (<https://digitalinsights.qiagen.com>) and trimmed and assembled using the default settings of the "Trim Reads" and "De novo assembly" tools, respectively. Sequences were aligned with BLAT v.36 (Kent, 2002) against the *Rattus norvegicus* RNOR 6.0 mitochondrial genome (Rat Genome Sequencing Project Consortium, 2004) to identify scaffolds corresponding to the mitochondrion. For each parent, the mitochondrion appeared to be contained in a single sequence, and subsequent BLASTN (Camacho et al., 2009) searches against the NCBI Nucleotide database (NCBI Resource Coordinators, 2016) on December 3, 2020 confirmed these findings.

2.5 | Read partitioning and haplotype assembly

PacBio reads were converted to the FASTA format using BAM2FASTA v.1.3.0 (<https://github.com/PacificBiosciences/bam2fastx>). Employing *k*-mers identified in the parental Illumina read data, CANU v.1.9 (Koren et al., 2018) was used to partition the PacBio reads of the hybrid by haplotype (and therefore species). The resulting species-specific reads were assembled separately using the default settings of CANU with an estimated genome size of 2.4 Gb.

2.6 | Genome polishing

BAM files containing species-specific reads identified by CANU were generated from the original PacBio subread BAMs and indexed using PBINDEX v.1.0.7 (<https://github.com/PacificBiosciences/pbbam>) to create input files for the PBMM2 aligner. Using the default settings of PBMM2 v.1.2.1 (<https://github.com/PacificBiosciences/pbmm2>; Li, 2018), the species-specific reads were aligned against the appropriate CANU reference. ARROW v.2.3.3 (<https://github.com/PacificBiosciences/GenomicConsensus>) was run using default settings to polish each assembly; due to the high memory requirements of this program, the PBMM2 BAMs and CANU reference FASTAs were split into individual files by contig, ARROW was run on each contig individually, and the resulting FASTAs were combined into a single file once the

polishing was complete. These polished assemblies were subjected to a second round of PBMM2 and ARROW to generate the final contigs.

2.7 | Identification and masking of repetitive elements

REPEATMODELER v.2.0.1 (Flynn et al., 2020) was run with the LTR structural search pipeline on each polished assembly to generate species-specific repeat libraries. Utilizing these libraries as well as the REPEATMASKER v.20181026 libraries from Repbase (Jurka, 1998), REPEATMASKER v.4.0.9 (<https://www.repeatmasker.org>) was used to mask repetitive elements in each assembly. As the resulting FASTAs were to be used for automated gene annotation, REPEATMASKER was run with the "-nolow" argument to avoid masking low complexity regions that could potentially comprise parts of genes (Tables S2 and S3).

2.8 | Processing and alignment of transcriptomic reads

Transcriptomic reads were supplemented with the RNA-seq reads used to annotate the previous ASM167557v1 draft release of the *N. lepida* genome (Campbell et al., 2016). Quality control and trimming of all RNA-seq reads was performed with TRIM GALORE! v.0.65 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), which utilized FASTQC v.0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and CUTADAPT v.2.10 (Martin, 2011). 99.55% of the reads were retained following TRIM GALORE! processing. Processed reads were aligned to each genome using the two-pass mode of STAR v.2.5.7a (Dobin et al., 2013), and merged into a single BAM file with SAMTOOLS v.1.9 (Li et al., 2009). See [Table S1](#) for mapping statistics.

2.9 | Automated gene prediction

Gene models on the repeat-masked references were predicted with BRAKER v.2.1.5 (Hoff et al., 2019). The STAR RNA-seq alignments, as well as all protein sequences from the OrthoDB v10 database (Kriventseva et al., 2019), were supplied to the BRAKER command line arguments "--bam" and "--prot_seq", respectively, and the application was run using the "--etpmode" and "--softmasking" arguments. Gene hints from the protein sequences and RNA-seq alignments were identified by the GENEMARK-ES v.4.59 suite (Brůna et al., 2020), and a subset of these predictions determined to be high quality based on the supporting OrthoDB and transcriptome evidence were selected by BRAKER for training. After aligning these predictions against each other with DIAMOND v.2.0.0 (Buchfink et al., 2015) to remove redundant models, these genes were used to train AUGUSTUS v.3.3.3 (Stanke et al., 2004) parameters for each species prior to gene prediction. Custom PYTHON scripts were used to reformat the resulting BRAKER GFF3 for sorting and validation with GENOMETOOLS v.1.6.1 (Gremme et al., 2013).

2.10 | Gene model filtering and functional annotation

Protein sequences were obtained from each species' BRAKER GFF3 and polished reference FASTA with scripts utilizing the BIOPYTHON v.1.76 libraries (Chapman & Chang, 2000). INTERPROSCAN v.5.51–85.0 (Jones et al., 2014), was run on each of these sequences, and all gene predictions lacking an InterPro group ID were removed from the BRAKER annotations. The filtered GFF3s were analysed and processed by the GFF3TOOLKIT v.2.0.3 (Chen et al., 2019) to resolve incomplete and spuriously duplicated models. All remaining protein sequences were aligned with BLASTP v.2.11.0+ (Camacho et al., 2009) against a database containing protein sequences from *Homo sapiens* release GRCh38 (Schneider et al., 2017), *Mus musculus* GRCm38.p6 (Mouse Genome Sequencing Consortium, 2002), *Peromyscus leucopus* PERLEU 2.1 (Long et al., 2019), *Peromyscus maniculatus* PMAN 2.1 and *Rattus norvegicus* RNOR 6.0 (Rat Genome Sequencing Project Consortium, 2004); with the exception of *P. leucopus*, all protein sequences were retrieved from Ensembl (Yates et al., 2019). When a protein prediction had a hit with an *E*-value under 10^{-5} , a description and gene name were retrieved from the corresponding annotation and added to the BRAKER GFF3 alongside all relevant InterPro group IDs and Gene Ontology categories.

2.11 | Annotation of P450 genes

Several sources of information were leveraged to identify and curate the P450 genes in each genome. We produced a set of gene models in BITACORA v.1.3 (Vizueta et al., 2020), which combines hidden Markov model (HMM) protein profiles and contig aware linear searches to annotate tandemly arrayed genes, using the default BITACORA settings and the Pfam PF00067 HMM profile. Additionally, we used the P450 amino acid sequence data set from Thomas (Thomas, 2007) to query our genomes using TBLASTN searches (v.2.11, *E*-value = 10^{-5}). Finally, we used HISAT2 v.2.2.1 (Kim et al., 2019) to conduct splice-aware RNA-seq alignments to each genome using the "--no-unal --no-mixed --no-discordant" options, and filtered the resulting alignment map files in SAMTOOLS v.1.9 (Li et al., 2009) to retain only the top alignment for each read pair. An additional GFF3 was also generated by aligning CDS sequences from the five animal genomes employed for functional annotation to each of the woodrat references using GMAP v.2020-06-01 (Wu & Watanabe, 2005) with "--min-trimmed-coverage" set to 0.8. The GMAP GFF3 was processed with the GFF3TOOLKIT (Chen et al., 2019) and sorted and validated with GENOMETOOLS (Gremme et al., 2013).

2.12 | Manual curation of cytochromes P450

The contigs, BLAST hits, RNA-seq alignments, and various gene model annotations were loaded into GENEIOUS v.10.2.6 (<https://www.geneious.com>) for manual curation. The P450 models from the combined BRAKER, GMAP, and BITACORA annotations were used for producing a final gene model with the following characteristics: adjacent exons were bounded

by AG/GT splice sites, exons included appropriate reading frames, and internal stop codons were absent. When RNA-seq data covered the entire model, the splice sites supported by the RNA-seq alignments were given priority. Putative P450s with early stops, or those with largely incomplete coding sequences based on TBLASTN searches, were marked as pseudogenes. Pseudogene designations included any BLAST hit that was near a functional gene or P450 island, and ranged from essentially complete sequences to hits matching single exons.

2.13 | Inference of syntenic P450 gene islands and copy number differences

Using the GENEIOUS v.10.2.6 viewer, we visualized the genomic organization of P450 2ABFGST and 3A genes for both *Neotoma* as well as an Old World rat (*R. norvegicus*) and New World *Peromyscus* mice, which are the sister genus to *Neotoma* (*P. maniculatus* for the CYP2ABFGST genes and *P. leucopus* for the CYP3A genes were chosen based on the completeness of the clusters in each species). Inference of expansions in *Neotoma* represents the most conservative comparison that can be made to its sister genus based on available resources. We also visualized nearby non-P450 genes to help establish regional synteny. To derive gene trees, the amino acid sequences of all functional genes within each subfamily were aligned in MAFFT v.7.45 (Katoh & Standley, 2013) using the G-INS-I algorithm and BLOSUM62 scoring matrix. We then used the "backtrans" function in TREEBEST v.1.9.2 (<http://treesoft.sourceforge.net>) to back translate the amino acid alignment to a protein-guided codon alignment of nucleotide sequences, and finally used the TREEBEST "best" function guided by the species tree (downloaded from <http://www.timetree.org>) with nodal support derived from 100 bootstraps. The models of each gene island and gene trees were then edited in ADOBE ILLUSTRATOR (Adobe Inc., San Jose, CA, USA), using both gene order and clustering in the gene tree to call putatively syntenic P450 clusters.

2.14 | Resolution of the genomic region containing the CYP2B array in *N. lepida*

While the CYP2B array was assembled as a single contig in the *N. bryanti* CANU assembly, the *N. lepida* array was significantly more fragmented, with CYP2B sequences identified across seven contigs. To resolve the region in *N. lepida*, haplotype-specific PacBio reads were aligned to the *N. lepida* assembly with MINIMAP2 v.2.17-r941 (Li, 2018), and only those mapping to the seven identified contigs were retained. Two CANU v.1.9 (Koren et al., 2018) assemblies were constructed with these reads: the first was generated using all reads (hereafter "AR"; genome size was left at 2.4 Gb and stopOnLow-Coverage was set to 0.7), while the second was generated using CANU's default coverage settings (hereafter "40x"; genome size was set to 19 Mb, the approximate size of the seven CYP2B-containing contigs). Each of these assemblies was polished with two rounds of PBMM2 v.1.2.1 (<https://github.com/PacificBiosciences/pbmm2>;

Li, 2018) and ARROW v.2.3.3 (<https://github.com/PacificBiosciences/GenomicConsensus>).

The 40× assembly appeared to resolve the entire region into two primary contigs: one 6.58 Mb and another 11.98 Mb in length. Though more fragmented overall, the AR assembly contained a single contig 13.63 Mb in length that, based on MUMMER v.3.23 (Kurtz et al., 2004) alignments (Figure S1 A,B), contained portions of both contigs from the 40× assembly. Using QUICKMERGE v.0.3 (Chakraborty et al., 2016) with “--length_cutoff” set to 1,000,000, the two 40× contigs were aligned against the 13.63-Mb AR contig to generate a single sequence 18.55 Mb in length. To polish this sequence, reads were first aligned with MINIMAP2 to the AR and 40× assemblies and only those mapping to the three contigs of interest were retained; these reads were then used for two rounds of polishing with PBMM2 and ARROW. Based on MUMMER alignments, the resulting sequence resolved the order and orientation of the largest four contigs in the initial CANU assembly (Figure S1C). The remaining three contigs could not be definitively placed and appeared to be repetitive sequences possibly arising from unresolved sequencing errors (Figure S1D). REPEATMASKER v.4.0.9 (<https://www.repeatmasker.org>) and the *N. lepida* REPEATMODELER v.2.0.1 (Flynn et al., 2020) library were used to mask repetitive elements on this sequence as previously described, and gene models from the initial assembly were transferred to this sequence using LIFTOFF v.1.6.1 (Shumate & Salzberg, 2021), with the manually curated P450s transferred using GENEIOUS v.10.2.6 (<https://www.geneious.com>).

2.15 | Annotation and curation of additional xenobiotic metabolizing families and transcription factors

For the annotation of the ATP-binding cassette transporter, flavin-containing monooxygenase, glutathione-S-transferase, sulpho-transferase and UDP-glucuronosyltransferase genes, along with the aryl hydrocarbon, pregnane X and constitutive androstane receptors (respectively encoded by the *AHR* and nuclear hormone receptor 112 and 113 genes—*NR112* and *NR113*), complete rodent protein sequences belonging to each family or transcription factor were obtained from the NCBI Protein database on 23 September 2021; sequences flagged as “partial” or “low quality” were omitted. Using the default settings of NCBI TBLASTN v.2.9.0 (Camacho et al., 2009) and GENOMETHREADER v.1.7.1 (Gremme et al., 2005), rodent sequences were aligned to each *Neotoma* genome to provide additional sources of evidence. Gene models from the *Mus musculus* and *Rattus norvegicus* annotations were also mapped to each *Neotoma* genome using the default settings of LIFTOFF v.1.6.1 (Shumate & Salzberg, 2021) to assist with annotation. The STAR RNA-seq alignments—which were converted to strand-specific big-Wig files with DEEPTOOLS v.3.5.1 (Ramírez et al., 2014)—along with the protein alignments, LIFTOFF models, BRAKER predictions and GMAP GFF3s were loaded into APOLLO v.2.6.5 (Dunn et al., 2019) for manual curation. Genes were required to have intact coding frames, priority was given to splice sites supported by the RNA-seq data

(provided the entire model had RNA-seq coverage), and models with partial or disrupted coding sequences were marked as pseudogenes. Homology data were additionally sourced from the Rat Genome Database (Smith et al., 2019) on 11 November 2021 to compile species counts for each gene family.

2.16 | Assessing genome completeness

Completeness for both genomes was assessed using the “genome” mode of BUSCO against the *Glirens_odb10* data set of 13,798 genes. We ran BUSCO v.4.1.4 (Seppey et al., 2019), which uses the gene predictions of both AUGUSTUS and TBLASTN, and the newer BUSCO v.5.0.0, which employs META-EUK (Levy Karin et al., 2020). The results of these searches were largely overlapping, but some genes were found by only one version. We combined the gene lists from each run to produce our final BUSCO metrics (Table 1).

JELLYFISH v.2.3.0 (Marçais & Kingsford, 2011) was used to tabulate *k*-mer counts of length 25 in the trimmed and corrected reads generated for each of the *Neotoma* assemblies. After discarding low frequency *k*-mers (those that appeared less than eight times for *N. bryanti* and 11 times for *N. lepida*), the genome size was estimated by multiplying each frequency value by the total number of *k*-mers present at that value, then dividing the sum of those numbers by the *k*-mer frequency value that appeared most often (24 for *N. bryanti* and 30 for *N. lepida*). Plots of the *k*-mer distributions for *N. bryanti* and *N. lepida* are shown in Figure S2.

2.17 | Genome scaffolding and synteny analysis

Contigs for both woodrats were aligned to the chromosome scaffolds of *Peromyscus leucopus* (Long et al., 2019), *P. maniculatus* and *P. nasutus* (https://www.dnazoo.org/assemblies/Peromyscus_nasutus), the closest relatives for which chromosome-level assemblies were available. Chromosome numbering and orientation are based on the *P. maniculatus* reference, with the corresponding chromosome sequences in *P. leucopus* and *P. nasutus* determined by MUMMER v.3.23 (Kurtz et al., 2004) alignments of those assemblies against the *P. maniculatus* reference. Due to the potential for misalignment due to repetitive elements, only *Neotoma* contigs exceeding 1 Mb in length with at least 100 kb of sequence uniquely aligned to a single chromosome were used in the scaffolding process. Contigs were aligned with the default settings of MUMMER v.4.0.0 beta 2 (Marçais et al., 2018), and assigned to the chromosome to which they had the greatest amount of sequence uniquely aligned. The orders and orientations of contigs along each chromosome were determined by the median position of their alignments, and were manually adjusted if discontinuities were evident when the MUMMER alignments were visualized (these discontinuities were almost universally large-scale inversions relative to the *Peromyscus* references). Once preliminary chromosome scaffolds were generated, the *N. bryanti* and *N. lepida* sequences were aligned against each other for validation as well as

to correct any outstanding contig placement issues. For both species, a total of 82 joins between adjacent contigs were made for the final chromosome scaffolds. These joins, each denoted by a stretch of 500N characters, account for the 41,000 bp of gap sequence present in each assembly (Table 1). Gene models were transferred to the new coordinates using custom scripts employing the BIOPYTHON v.1.76 libraries (Chapman & Chang, 2000).

After scaffolding, syntenic regions between each *Neotoma* genome and *P. leucopus* and *P. maniculatus* were identified using BLASTALL v.2.2.26 (Altschul et al., 1990) protein alignments with an *E*-value of 10^{-20} and the default settings of SYNIMA (GitHub release a0bc445) (Farrer, 2017). MUMMER plots against the *Peromyscus* genomes are available for all *N. bryanti* chromosomes as Figures S3–S5, and all *N. lepida* chromosomes as Figures S6–S8. MUMMER plots between the *Neotoma* chromosome scaffolds are available in Figures S9 and S10. Information on the relative order and orientation of contigs for each chromosome is contained in Table S4.

2.18 | Estimating nuclear and mitochondrial divergence

The *N. bryanti* and *N. lepida* chromosome scaffolds were aligned to each other using the NUCMER program of MUMMER v.3.23 (Kurtz et al., 2004), and DELTA-FILTER was run on the alignments to retain only regions that had one-to-one mappings between both species. Variants in these regions were then called using the MUMMER SHOWSNPS program, and metrics were calculated using a custom PYTHON script. The same process was performed for the mitochondrial scaffolds. Complete statistics for both the nuclear and mitochondrial genomes are contained in Table S5.

TABLE 1 Assembly metrics and BUSCO assessments for *Neotoma bryanti* and *Neotoma lepida*

Metric	<i>Neotoma bryanti</i>	<i>Neotoma lepida</i>
Assembly size (bp)	2,600,174,201	2,612,755,627
Contig count	2397	2421
Contig N50 length (bp)	39,917,500	52,364,606
Longest contig (bp)	151,130,156	139,341,509
Shortest contig (bp)	1077	1059
Chromosome scaffold size (bp)	2,388,581,836	2,399,855,743
Chromosome scaffold count	24	24
Chromosome scaffold N50 length (bp)	111,123,070	111,355,628
Longest chromosome scaffold (bp)	187,705,395	189,504,864
Shortest chromosome scaffold (bp)	45,124,132	44,361,528
Chromosome scaffold gaps (bp)	41,000	41,000
BUSCO completeness (Gfires – 13,798 BUSCOs)	97.62% (13,469)	97.63% (13,471)
Complete, single-copy	96.46% (13,309)	96.42% (13,304)
Complete, duplicated	1.16% (160)	1.21% (167)
Fragmented	0.34% (47)	0.26% (36)
Missing	2.04% (282)	2.11% (291)

3 | RESULTS AND DISCUSSION

3.1 | Read binning produces highly contiguous and complete assemblies

The read lengths provided by recent advances in sequencing technology, combined with the trio binning approach, yielded an almost perfect separation of parent-of-origin sequencing data from the F_1 hybrid. After removing all PacBio reads considered too short (<1 kb, which accounted for 1.97 Gb, or 0.25% of the total), CANU assigned 49.46% (389.60 Gb) of reads to the maternal *N. bryanti* haplotype and 50.16% (395.14 Gb) to the paternal *N. lepida* haplotype. Only 0.13% (1.05 Gb) was unassignable. This marks a slight advancement over that reported for trio binning within *Bos taurus* (49.3 and 49.6% assigned to each haplotype, respectively) (Koren et al., 2018), and is in line with the results reported for the recent cross-species *Bos* (Rice et al., 2020) and feline (Bredemeyer et al., 2020) assemblies. The highly accurate partitioning of trio binning yielded minimal loss of usable sequencing data, further demonstrating the promise this technique holds for generating genomes from hybridized taxa.

Though well over 2000 contigs were produced for each woodrat reference, the N50 values indicate that the vast majority of each assembly lies within large, contiguous sequences (Table 1). Furthermore, although <5% of contigs exceeded 1 Mb (110 for *N. bryanti* and 107 for *N. lepida*), these sequences contained over 91% of each 2.6-Gb assembly. Of particular note were the extremely long contigs generated for each species; with lengths exceeding 100 Mb, these sequences probably represent most (or all) of a chromosome.

BUSCO analysis of the assemblies indicated that they were remarkably complete, with *N. bryanti* containing 97.62% and

N. lepida possessing 97.63% of the 13,798 single-copy orthologues present in the Glres 10 data set (Table 1). Additionally, both assemblies contained few partial or multicopy BUSCOs, indicating that fragmentation and sequence duplication were minimal. We investigated this small number of multicopy BUSCOs to see if they contained an enrichment of xenobiotic metabolizing genes, but found relatively few in this category. This is not surprising, however; BUSCO is designed to locate highly-conserved, single-copy orthologues (Seppey et al., 2019), and as many xenobiotic metabolizing subfamilies have members with a high degree of sequence similarity—a feature which complicates accurate orthologue identification—their underrepresentation in the BUSCO databases is not unexpected. Further bolstering the completeness of the references, the total assembly lengths were similar to size estimates based on *k*-mer counts from the PacBio sequencing data (2.72 and 2.66 Gb for *N. bryanti* and *N. lepida*, respectively), with the assembled and estimated values differing by less than 5%. Together, these results indicate that the vast majority of the nuclear genome for both species is contained in the assemblies.

Similar results were found for the mitochondrial genomes reconstructed from the parental short read data. Each mitochondrial scaffold—16.7 kb for *N. bryanti* and 16.3 kb for *N. lepida*—was very close in size to the 16.3-kb mitochondrial assemblies for *R. norvegicus* (Rat Genome Sequencing Project Consortium, 2004) and *M. musculus* (Mouse Genome Sequencing Consortium, 2002). These completeness findings were further strengthened with BLASTN (Camacho et al., 2009) searches against the NCBI Nucleotide database (NCBI Resource Coordinators, 2016), which found query coverages of 97% and 99% for *N. bryanti* and *N. lepida*, respectively, for the closely related *N. magister* and *N. mexicana* mitochondrial genomes.

3.2 | Scaffolding indicates most chromosomes were assembled from few contigs

As no chromosome-level *Neotoma* assemblies exist, we used alignments against the genomes of *Peromyscus leucopus*, *P. maniculatus* and *P. nasutus* to scaffold our contigs into chromosomal sequences. Using the 24 chromosome scaffolds from the *Peromyscus* assemblies as references, we scaffolded 96.36% of contigs exceeding 1 Mb for *N. bryanti* (106/110), and 99.07% (106/107) for *N. lepida* (Figure 2a,b). The scaffolded chromosomes resulting from this approach contained the vast majority of each species' genome, comprising 91.86 and 91.85% of the *N. bryanti* and *N. lepida* assemblies, respectively (Table 1). Though the number of chromosomes in *Neotoma* (1N = 26 chromosomes in *N. lepida*) may be slightly higher than in *Peromyscus* (Mascarello & Hsu, 1976), given the close phylogenetic proximity of these genera, our scaffolding approach based on the 24 *Peromyscus* chromosomes provides a reasonable approximation of each *Neotoma* species' chromosomal architecture.

Among the assembled chromosomes, five were composed of single contigs; these were chromosomes 4 and 19 for *N. bryanti* and 12, 13 and 19 for *N. lepida* (Figure 2a,b). Further, of the

chromosomes scaffolded for both species, the vast majority (23/24 for *N. bryanti* and 22/24 for *N. lepida*) were assembled from eight or fewer contigs—only the X chromosome for *N. bryanti* (23 contigs) and chromosomes 1 and X for *N. lepida* (12 and 17 contigs, respectively) were composed of more (Table S4). Given that repetitive elements comprise large portions of the X chromosome in mammals (Ross et al., 2005) and can be notoriously difficult to assemble, the fragmentation of this chromosome in both assemblies is not surprising. Nevertheless, the selection of a female hybrid offspring for sequencing enabled us to reconstruct the X chromosome for both species.

3.3 | Large-scale chromosome structure and synteny are conserved

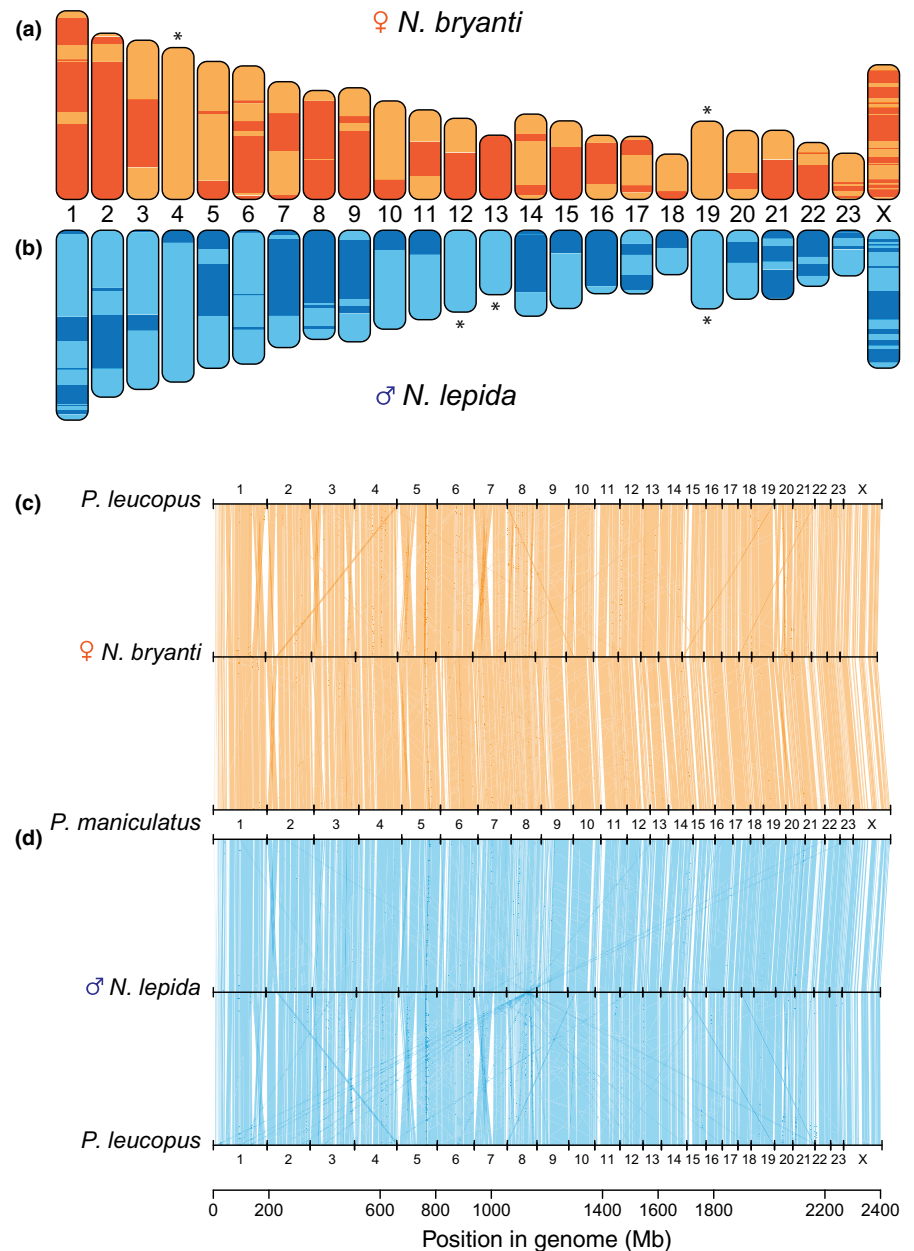
Alignments of the scaffolded *Neotoma* chromosome sequences to the three available *Peromyscus* genomes reveal that the majority of the chromosomal sequence is present in the same order and orientation in both genera (Figures S3–S8). While a number of structural rearrangements (primarily inversions) are present, we see no evidence to suggest translocation events between chromosomes. Furthermore, the number of inversions detected might be inflated. Very few of these structural features are present in all three *Peromyscus* references (Figures S3–S8), leading to the possibility that some inferred inversions might be assembly artefacts resulting from the different scaffolding approaches taken for each *Peromyscus* genome. Synteny findings, too, are consistent with the alignment results, with gene positions strongly conserved between the *Neotoma*, *P. leucopus*, and *P. maniculatus* genomes (Figure 2c,d).

Though our alignment-based approach cannot rule out large-scale structural differences, individual contig alignments between *N. bryanti* and *N. lepida* lend no support to their existence (Figures S9 and S10). This is intriguing given the chromosome variation previously observed within *Neotoma*. Chromosome counts range from 1N = 19 in *N. phenax* to 1N = 28 in *N. fuscipes*, with 1N = 26 being the most common for this genus (Baker & Mascarello, 1969); research on *N. lepida* has also found evidence for chromosomal polymorphisms within this species that vary by population (Mascarello & Hsu, 1976). The lack of such structural changes, however, may be instrumental in maintaining the viability and fertility of hybrid offspring.

3.4 | Nuclear and mitochondrial sequence divergence between *N. bryanti* and *N. lepida*

Sequence divergence was 1.27% between the *N. bryanti* and *N. lepida* nuclear genomes (Table S5), which agrees with an estimated 1.5-million-year divergence (Galewski et al., 2006; Patton et al., 2007). For the mitochondrial genome, divergence was 6.96%; this is not substantially different than the 9% divergence previously found for cytochrome *b* alone (Patton et al., 2007).

FIGURE 2 Contig scaffolding and synteny results for both *Neotoma* genomes. (a,b) Visualization of contigs scaffolded into chromosomes for *Neotoma bryanti* (a) and *N. lepida* (b). Alternating colours denote individual contigs, and chromosomes indicated with an asterisk were assembled in their entirety as a single contig. (c,d) Identification of syntenic gene alignments by SYNIMA for the *N. bryanti* (c) and *N. lepida* (d) autosome scaffolds 1–23 and X chromosome scaffold against the *Peromyscus leucopus* and *P. maniculatus* genomes. Note that while there are a number of instances of cross-alignments among the chromosomes (probably due to cross-mapping between genes in duplicated families), the positions of the majority of genes are strongly conserved between *Neotoma* and *Peromyscus*



3.5 | Gene counts and repetitive element annotations are similar to model rodents

A total of 22,444 protein coding genes were identified for *N. bryanti*, and 21,453 were identified for *N. lepida*. These numbers align with the 22,469 and 23,139 protein coding genes in the current *M. musculus* (Mouse Genome Sequencing Consortium, 2002) and *R. norvegicus* (Rat Genome Sequencing Project Consortium, 2004) annotations, respectively. REPEATMASKER results are also in agreement with *M. musculus* (<http://www.repeatmasker.org/species/mm.html>) and *R. norvegicus* (<http://www.repeatmasker.org/species/rn.html>), with 40.62 and 41.11% of the *N. bryanti* and *N. lepida* assemblies, respectively, comprised of repetitive elements (Tables S2 and S3).

3.6 | Dynamic evolution of cytochrome P450 gene islands and the genomic basis of toxin tolerance

After confirming the completeness of the *Neotoma* assemblies and annotations, we examined several P450 subfamilies (CYP2A, CYP2B, CYP3A) because of their role in xenobiotic metabolism (Thomas, 2007). All three subfamilies showed clear signs of dynamic evolution and lineage-specific expansion, in contrast to the relative stability of CYP2F, CYP2G, CYP2S, and CYP2T genes, which are in the same P450 tandem array as the CYP2A and CYP2B subfamilies (Figure 3a). *R. norvegicus* and *P. maniculatus* have three CYP2A genes, which have expanded to seven and six genes in *N. bryanti* and *N. lepida*, respectively (Table 2, Figure 3a). Based on the gene tree (Figure 3c), separate duplication events of CYP2A2-like and

CYP2A3-like genes are responsible for the increase in CYP2A counts. Following the relationships among species, the most parsimonious interpretation of structural changes in CYP2A appears to be expansion in *Neotoma* rather than copy loss in other genera.

The pattern of expansion is even more remarkable in the CYP2B subfamily (Figure 3b). CYP2B copy number variation among

Neotoma species was previously documented using quantitative PCR and cDNA cloning approaches (Kitanovic et al., 2018; Malenke et al., 2012), and we can now start to understand the spatial organization of this variation. We infer the evolution of a novel gene island containing only tandemly arrayed CYP2B gene copies in New World rats and mice (*Neotoma* and *Peromyscus*). Typically, CYP2B genes are

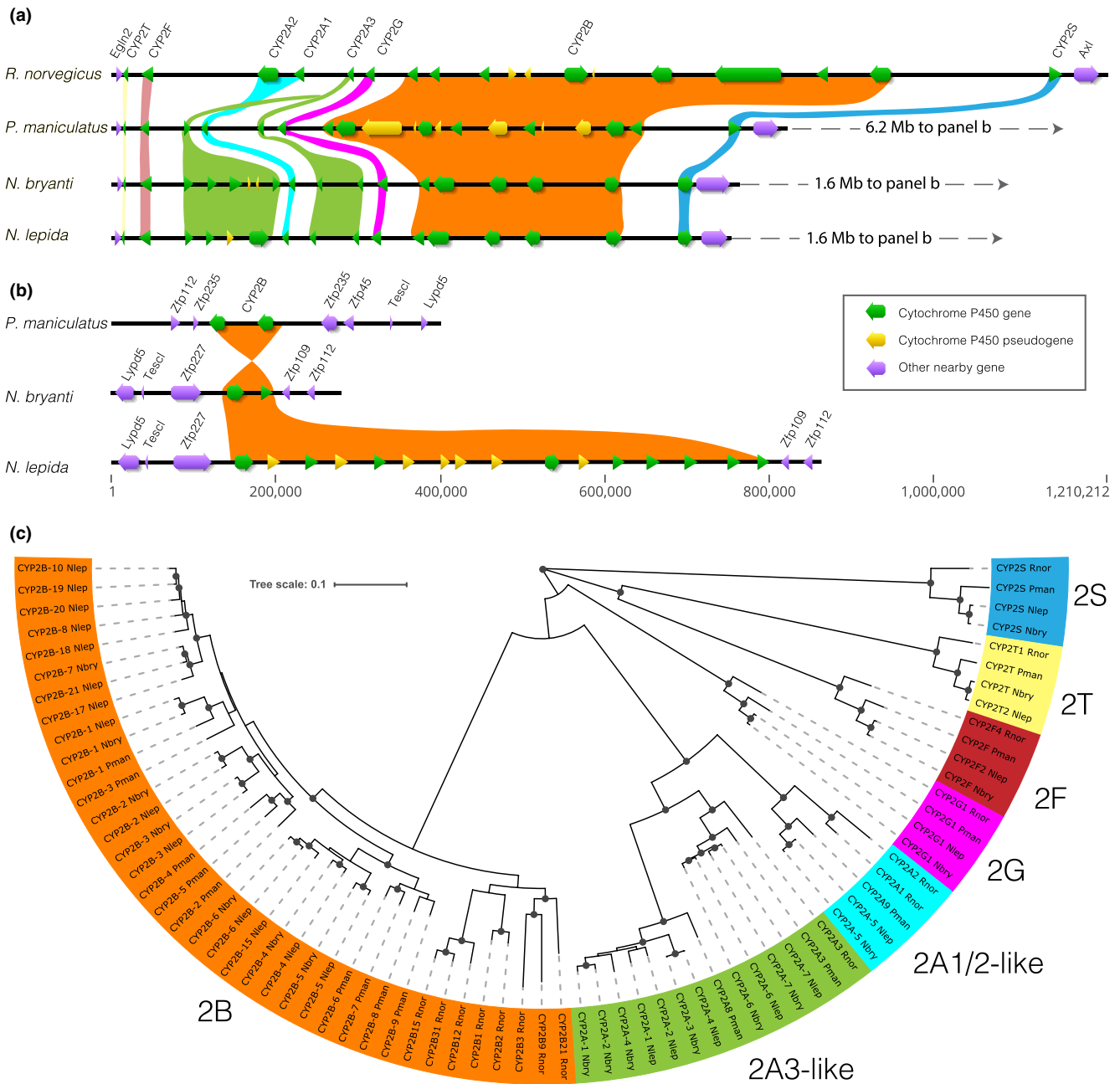


FIGURE 3 Genomic architecture and evolution of the CYP2ABFGST gene islands in four rodents. Visualization of the contigs containing these genes was based on annotations for the Norway rat (*Rattus norvegicus*; mRatBN7.2) and deer mouse (*Peromyscus maniculatus*; HuPman2.1), and manual annotation of these regions for *Neotoma bryanti* and *N. lepida*. CYP2 families are colour coded similarly across all figure panels. (a) a highly conserved gene island is bounded by *Egn2* and *Axl* and retains subfamily order in *Neotoma* and *Peromyscus*; gene order is also similar to that of other vertebrates. (b) a second gene island composed of variable numbers of CYP2B genes arose in the New World rodents only, and underwent further expansion in *N. lepida*. (c) a tree of the inferred functional genes from each species was used to determine gene cluster identity/orthology. Circles at nodes indicate bootstrap values of 70% or higher

TABLE 2 Cytochrome P450 subfamily gene counts

P450 subfamily	<i>Rattus norvegicus</i>	<i>Peromyscus</i> sp.	<i>Neotoma bryanti</i>	<i>Neotoma lepida</i>
CYP2A	3	3 ^{a,b}	7	6
CYP2B	8	9 ^a	7	14
CYP3A	6	13 ^b	18	16

^a*Peromyscus maniculatus* used for gene counts.

^b*Peromyscus leucopus* used for gene counts. Subfamily completeness varies between the two available *Peromyscus* genomes, with neither having all three clusters intact.

found only between the single-copy CYP2G and CYP2S genes in the CYP2ABFGST gene array (Figure 3a,b). This is the arrangement for *Mus musculus*, humans and other primates (Hoffman & Hu, 2007; Thomas, 2007). However, examination of this gene array in other systems has shown dynamic rearrangement via duplication, deletion, and inversion within a genomic region bounded by the *Egl*n2 and *Axl* genes (Hu et al., 2008). Similarly, New World rodents also show variation in the arrangement of CYP2B genes within the ancestral location of this array, evidenced by two extra functional copies and several pseudogenes in *P. maniculatus* compared to woodrats, and the inverted orientation of the CYP2B gene closest to CYP2S in woodrats (Figure 3a). Within *Neotoma*, we find a novel gene island outside the bounds of the ancestral array, comprised solely of variable numbers of CYP2B genes (Figure 3b). Based on the relationships among these copies, the novel island probably resulted from a duplication and translocation of the CYP2B gene closest to CYP2S to a location more than 1 Mb away on the same chromosome, bounded by genes encoding zinc-finger proteins, *Tescl* and *Lypd5*.

The novel CYP2B gene island consists of duplicates of sequences most similar to CYP2B37 (Figure S11), while CYP2B35 and CYP2B36 appear to be contained within the bounds of the ancestral island (Wilderman et al., 2014). We identified and named CYP2B35, 36, and 37 in concordance with previous work on *Neotoma lepida* (Malenke et al., 2012; Wilderman et al., 2014). In addition to the unique position of the CYP2B37 genes (Figure 3b), their proteins show unique enzymatic activity and substrate binding relative to CYP2B35 and CYP2B36 (Wilderman et al., 2014). It is possible that translocation to this new position facilitated the process of neofunctionalization of these genes, as has been proposed in other systems (Deng et al., 2010; Wen et al., 2006). Additionally, there is copy variation between *N. bryanti* and *N. lepida* (Figure 3b), with two functional copies in *N. bryanti* and nine functional copies plus seven pseudogenes in *N. lepida*. These findings, in combination with the known functional uniqueness of CYP2B37 proteins, are consistent with the hypothesis that evolution within unstable gene islands is a source of adaptive novelty in the arms-race between herbivores and the plants on which they feed (Thomas, 2007; Wen et al., 2006; Wilderman et al., 2014).

The pattern observed in the CYP3A subfamily was similar to the dynamic evolution apparent in CYP2B (Figure 4), and contrasted with the relative conservatism of the CYP genes involved in biosynthesis. First, all CYP3A genes are found on a single chromosome (chromosome 12 in *R. norvegicus*, chromosome 23 in *Peromyscus* and our

Neotoma assemblies). In *R. norvegicus*, the two CYP3A gene islands are approximately 7 Mb apart (Figure 4a). The first island is small and contains the CYP3A9-like genes, which play a role in steroid hormone synthesis (Xue et al., 2003). This island showed general conservation among rodent species, with two functional copies in *R. norvegicus*, two in *P. leucopus*, three in *N. bryanti*, and one plus two pseudogenes in *N. lepida*. Moreover, these genes clustered together in the gene phylogeny, and this conservation may reflect the homeostatic roles CYP3A9 genes play.

In contrast, the remaining CYP3A gene islands all have evolved dynamically among the *Peromyscus* and *Neotoma* species included here. The second of two gene islands in *R. norvegicus* contains four functional CYP3A genes and is bounded by several non-P450 genes that appear in the other taxa. However, between CYP3A islands syntenic with the *R. norvegicus* island lies another island that appears to be unique to the New World rodents (Figure 4b). This array is bounded by *Vmn2r* and *Anhx*, and contains nine functional copies of CYP3A in *P. leucopus* (CYP3A13 in RefSeq) and five in each woodrat. Using CYP5A1 genes as an outgroup, we found that the island containing the CYP3A13-like sequences is the sister group of the other CYP3A genes (Figure 4d). Given this relationship, the CYP3A13-like genes can be inferred to be duplicated from the common ancestor of the CYP3A11 and CYP3A9-like genes, both of which are present in New and Old World rodents. This raises the intriguing possibility that the CYP3A13-like genes were present in the ancestor of all rodents studied herein, and lost in the lineages leading to *R. norvegicus*. Alternatively, a dynamic history of duplication with gene conversion may be obscuring these deeper relationships within the gene tree, as suggested by the low branch support for the ancestral node leading to the CYP3A13-like genes. Broader comparative analyses with other highly contiguous rodent genomes will be needed to address the order of these large duplication events involving the CYP3A genes.

On chromosome 23, we encountered CYP3A11-like genes similar in number and sequence to their putative orthologues in *R. norvegicus* (Figure 4c). In *Mus*, CYP3A11 is highly expressed in the liver and neurons, where it protects against a wide variety of bioactive compounds and cytotoxic chemicals (Hagemeyer et al., 2003). However, the arrangement of CYP3A11-like gene copies changes drastically with the inclusion of the two *Neotoma* species; the inferred ancestral single gene island is instead two distinct islands separated by the non-P450 gene *Usp12*. The novel fourth CYP3A gene island in *Neotoma* appears to have arisen, in part, due to duplication and inversion of a large segment of DNA. The *Zscan25*

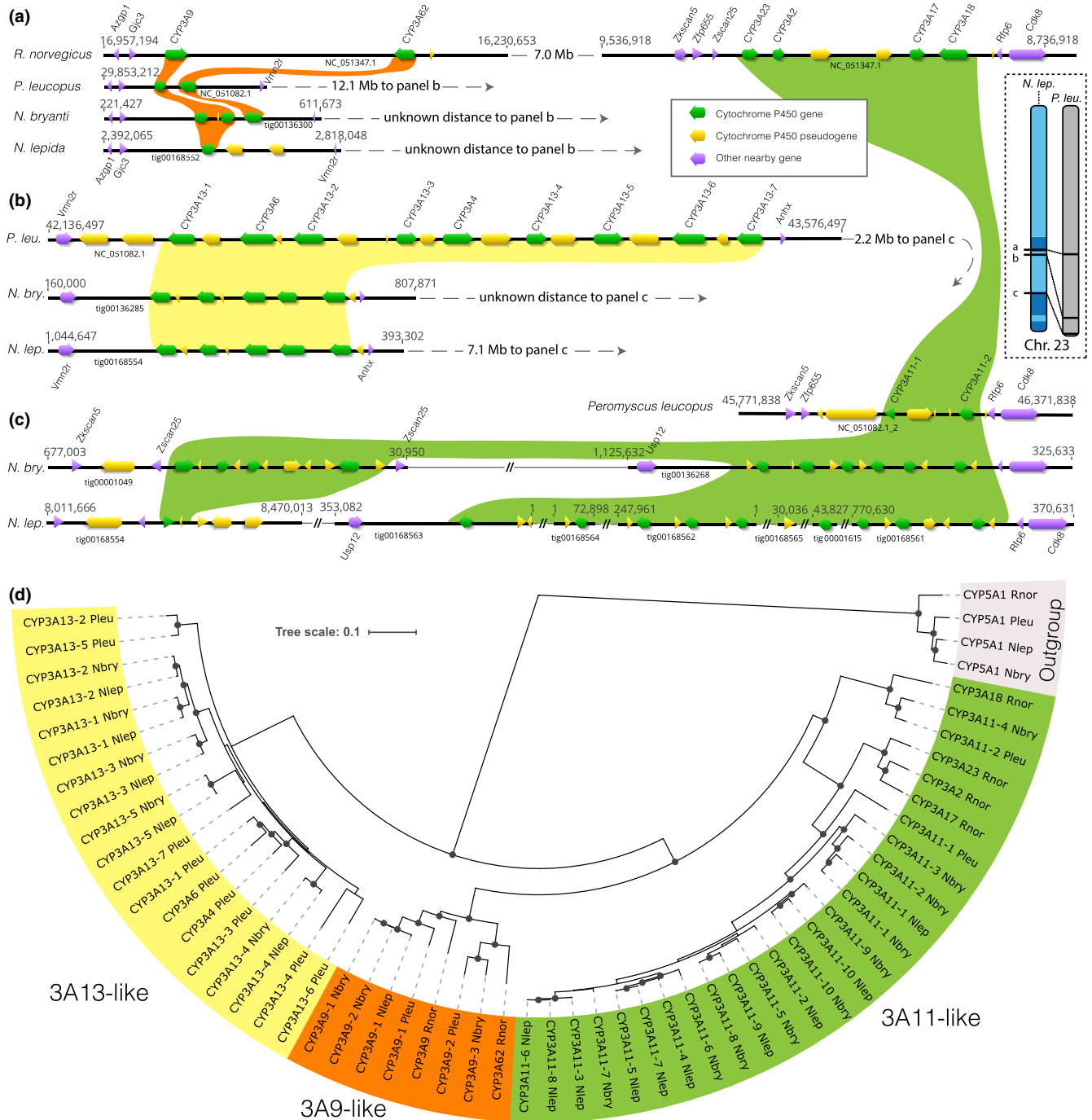


FIGURE 4 Genomic architecture and evolution of the CYP3A gene islands in four rodents. Visualization of the contigs containing these genes was based on existing annotations for the Norway rat (*Rattus norvegicus*; mRatBN7.2) and white-footed mouse (*Peromyscus leucopus*; UCI_PerLeu_2.1), and manual annotation of these regions for *Neotoma bryanti* and *N. lepida*. (a) Organization of *R. norvegicus* CYP3A genes on chromosome 12 (top contig), as well as the largely conserved CYP3A9-like genes found in *P. leucopus* and both *Neotoma*. (b) a second gene island has no clear orthology with a region in *R. norvegicus*. (c) the downstream *R. norvegicus* genes in (a) are related to a third downstream gene island in *P. leucopus* that is further divided into a third and fourth gene island in *Neotoma*. (d) a gene tree of the functional genes from each species was used to determine gene cluster identity/orthology, and is colour coded similarly across all figure panels. Circles at nodes indicate bootstrap values of 70% or higher. The inset (top right) indicates the approximate locations of panels (a), (b) and (c) on chromosome scaffold 23 for *N. lepida* (blue; 45.6 Mb in length) and *P. leucopus* (grey; 46.5 Mb in length). Alternating colours denote the individual contigs comprising chromosome scaffold 23 in *N. lepida*

gene, which bounds one side of this gene island in both *R. norvegicus* and *P. leucopus*, appears on each end of the novel island of CYP3A11-like genes in *N. bryanti* (Figure 4c), and a large CYP3A

pseudogene now separates *Zkscan5* and *Zscan25* on the 5' side of the array. Together, these duplicated genes and novel islands account for the increase in CYP3A genes in New World rodents

compared to the six functional copies of *CYP3A* in *R. norvegicus* (Table 2), with woodrats having the largest number of functional copies among the species examined.

3.7 | Expansions in other xenobiotic metabolizing families, but not transcription factors, may play a role in novel toxin degradation

We also explored the dynamics of several phase I, II and III biotransformation families, as well as key transcription factors, to further our understanding of xenobiotic metabolism in *Neotoma*. As many of these families are not well-studied within Rodentia outside of *M. musculus* and *R. norvegicus*, we restricted all comparisons of *N. bryanti* and *N. lepida* to these two rodent species. Therefore, it is possible that a number of the expansions discovered may be present not only in the genus *Neotoma*, but in other closely related cricetid species as well.

Flavin-containing monooxygenases (FMOs), another phase I family, exhibit a different pattern compared to the P450s. Exceptionally efficient at oxygenating substrates into more polar metabolites (Katzung, 2018), FMOs are highly conserved across the tree of life (Huang et al., 2021) and have similar copy numbers across mammalian lineages (Koukouritaki et al., 2002). Consistent with this conservation, we found no evidence for expansion in this family, with all putatively functional FMOs in mouse and rat (*FMO1–6*) present as single copies in both *Neotoma* species (Table 3, Table S6). Additionally, intact models for *FMO9*, 12 and 13, which appear to have complete coding frames but nevertheless may be pseudogenes in *M. musculus* (Hernandez et al., 2004), were located in each woodrat genome.

Among the phase II enzymes, which conjugate various chemical groups to xenobiotics (Katzung, 2018), we explored the three enzyme classes responsible for the vast majority of phase II reactions in animals: glutathione-S-transferases (GSTs), sulphotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs) (Daniel, 1993; Dutton, 1980; Runge-Morris & Kocarek, 2009). For the GSTs, which neutralize substrates through the addition of a glutathione group (Katzung, 2018), we observed modest expansions compared to mouse and rat. Out of the six cytosolic subfamilies (Nebert & Vasiliou, 2004), these duplications were confined to just two: GST alpha (GSTA) and mu (GSTM). Among the remaining subfamilies—omega (GSTO), pi (GSTP), theta (GSTT) and zeta (GSTZ)—no expansions or contractions were evident (Table 3, Table S7). Though evolutionarily distinct from the cytosolic GSTs, we found similar results for the kappa (GSTK), microsomal (MGST) and prostaglandin E synthase genes (Nebert & Vasiliou, 2004). GSTK and MGST numbers matched those of mouse and rat, while the prostaglandin E synthase genes appeared to have one duplication (*PTGES*) in *N. lepida*.

This same pattern extended to the SULT family, a group of enzymes that metabolize compounds through sulphation (Katzung, 2018), where only a few modest expansions were discovered (Table 3, Table S8). Three subfamilies, SULT1, 2 and 3, appeared to have a few duplications in *N. bryanti* and *N. lepida*, while

the SULT4, 5 and 6 numbers agreed with those of *M. musculus* and *R. norvegicus* (Alnouti & Klaassen, 2006; Gamage et al., 2006; Smith et al., 2019). Additionally, the two *PAPSS* genes, which synthesize the sulphate group donor for all SULT enzymes (Katzung, 2018), were present as single copies in both *Neotoma* genomes, indicating they had not undergone expansion. As the reactions catalysed by the GST and SULT families can be metabolically more expensive than those of the phase I enzymes (Klaassen, 2001) and result in the loss of conjugates that may be limiting (e.g., sulphur, amino acids), our findings of modest duplications in these two families are not surprising from an efficiency standpoint.

In stark contrast to the other phase II enzymes, we found profound expansions in the UGT genes, a class of enzymes that catalyse the conjugation of a metabolite with glucuronic acid (Katzung, 2018) (Table 3, Table S9). Though the additions were limited to a single subfamily, UGT2B, the numbers of apparently intact genes, 20 for *N. bryanti* and 27 for *N. lepida*, dwarf the seven in mouse (Buckley & Klaassen, 2007) and rat (Smith et al., 2019). While we were not able to assemble these genes on a single contig for either *Neotoma* species, MUMMER alignments indicate that this is a single genomic region on chromosome 10 that appears to have undergone recent and substantial tandem duplication. Interspersed among the intact UGT2B genes were 18 and 26 pseudogenes for *N. bryanti* and *N. lepida*, respectively, demonstrating an unusually high degree of turnover. Outside of UGT2B, numbers for the remaining UGT subfamilies (UGT1A, 2A and 3A) were all in line with *M. musculus* and *R. norvegicus*, though an extra UGT3A gene was found in *N. bryanti*. As a family, UGTs are exceptionally versatile in substrate recognition (Klaassen, 2001), and the UGT2B subfamily in particular is involved in the metabolism of a wide range of both xenobiotic and endogenous metabolites (Meech et al., 2019). Notably, increased glucuronidation levels have been previously reported for *N. lepida* individuals fed high amounts of *Larrea tridentata* (Haley et al., 2008; Mangione et al., 2001), so it is a distinct possibility that the UGT2B expansions observed in both genomes play a role in enabling these woodrats to subsist on a diet rich in creosote bush. In addition, biotransformation of xenobiotics by P450s is often followed with glucuronidation, and a concomitant expansion of UGTs and P450s makes sense biologically.

Lastly, as part of our efforts to better understand woodrat phase III detoxification—the efflux of xenobiotic compounds across cellular membranes—we also explored the ATP-binding cassette (ABC) transporter family (Ishikawa, 1992; Li et al., 2015). Similar to the UGTs, we observed substantial expansion confined to a single subfamily, in this case ABCG (Table 3, Table S10). This subfamily has broad substrate recognition, but is primarily involved in the transport of hydrophobic compounds (both endogenous and xenobiotic) including lipids, bile salts and sterols (Kerr et al., 2011). The expansion was driven by the proliferation of a single member of the ABCG subfamily on chromosome 10: *ABCG3*. *ABCG3* genes appear to be unique to rodents and have a distinct ATP-binding domain (Mickley et al., 2001), but are otherwise poorly studied. The region encompassing these genes was assembled in its entirety for both *Neotoma* species, and much like the *CYP2B* and *UGT2B* subfamilies, tandem duplication with a

Gene family/subfamily	<i>Mus musculus</i>	<i>Rattus norvegicus</i>	<i>Neotoma bryanti</i>	<i>Neotoma lepida</i>
FMO (phase I)	9	8	9	9
GST (phase II)	22	22	29	28
GSTA	5	6	10	10
GSTM	7	8	10	9
GSTO	2	2	2	2
GSTP	3	1	2	2
GSTT	4	4	4	4
GSTZ	1	1	1	1
SULT (phase II)	21	14	28	24
SULT1	6	7	9	7
SULT2	9	4	10	9
SULT3	2	0	5	4
SULT4	1	1	1	1
SULT5	1	1	1	1
SULT6	2	1	2	2
UGT (phase II)	12	11	26	32
UGT1A	1	1	1	1
UGT2A	2	2	2	2
UGT2B	7	7	20	27
UGT3A	2	1	3	2
ABC (phase III)	53	57	66	65
ABCA	16	16	15	15
ABCB	12	12	13	16
ABCC	11	11	11	11
ABCD	4	4	4	4
ABCE	1	1	1	1
ABCF	3	3	3	3
ABCG	6	10	19	15

TABLE 3 Intact gene and subfamily counts for additional phase I, II and III biotransformation families

high degree of gene turnover is evident, suggesting relatively recent expansion. Modestly increased gene counts were also observed in the ABCB subfamily, which plays a role in xenobiotic metabolism in mammals (Sarkadi et al., 2006); *ABCB3/TAP2* was duplicated in both species, and extra copies of *ABCB1* and *ABCB2/TAP1* were present in *N. lepida*. All ABCA genes were present in both *Neotoma* genomes, though unlike *M. musculus* and *R. norvegicus*, only a single copy of *ABCA8* was present. The remaining ABC subfamilies (C, D, E and F) each had numbers matching those of mouse and rat, and no expansions were evident.

Finally, we also investigated whether or not duplication among transcription factors may play a role in regulating the response to xenobiotic metabolism, but found this was unlikely to be the case. The aryl hydrocarbon receptor (*AHR*) and nuclear hormone receptors 112 and 113 (*NR112* and *NR113*; these encode the pregnane X and constitutive androstane receptors, PXR and CAR, respectively), which are the primary transcription factors involved in coordinating the expression of multiple biotransformation genes in mammals

(Tolson & Wang, 2010; Wang & LeCluyse, 2003; Zhang et al., 2008), were intact in each sequenced genome and present only as single copies.

4 | CONCLUSIONS

Our study contributes the first nearly complete, thoroughly annotated genomes of the genus *Neotoma*. Given the complexity, repetitiveness and high degree of turnover in several biotransformation families, and the P450 islands in particular, long-read DNA sequencing technology was crucial to our ability to accurately reconstruct the evolutionary history of the genes studied herein. These genomes now provide the basis for understanding aspects of adaptive evolution in these and related species, and in particular, the dynamics of three specific biotransformation families involved in diet-related adaptations: P450 2B and 3A, UGT2B and ABCG. As anthropogenic climate change over the coming decades is expected to lead to vast

alterations in vegetation (Rosenzweig et al., 2007)—and therefore drastic changes in mammalian herbivore diets—woodrats can provide insight into the physiological and genetic factors that are crucial for survival and species conservation. These new genomic resources lay the groundwork for future studies into the adaptability of this unique genus (and vertebrate herbivores in general), and the success and efficiency of the trio binning method should encourage other researchers studying hybridizing species to strongly consider utilizing this approach.

AUTHOR CONTRIBUTIONS

M. Denise Dearing and Michael D. Shapiro designed the study, with input from Marjorie D. Matocq and Thomas L. Parchman. Teri J. Orr performed fieldwork, animal husbandry and tissue dissection. Robert Greenhalgh performed genome assembly and generated automated annotations. Robert Greenhalgh and Matthew L. Holding performed genomic analyses. Robert Greenhalgh and Matthew L. Holding curated gene models with input from James B. Henderson. Robert Greenhalgh and Matthew L. Holding wrote the manuscript with input from all authors.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The CLC assemblies, Canu assemblies, chromosome scaffolds and accompanying annotations for *Neotoma bryanti* and *Neotoma lepida* have been deposited at the Centre for Open Science (<https://doi.org/10.17605/osf.io/xck3n>). The genomic and RNA-seq reads generated for this work have been deposited at the SRA under BioProject PRJNA818341. The custom scripts used throughout this project are available at <https://github.com/robertgreenhalgh/trio-scripts>

OPEN RESEARCH BADGES



This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at the SRA (BioProject PRJNA818341), the Centre for Open Science (<https://doi.org/10.17605/osf.io/xck3n>) and <https://github.com/robertgreenhalgh/trio-scripts>.

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